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CARCINOGENESIS OF NITRATED TOLUENES AND BENZENES SKIN
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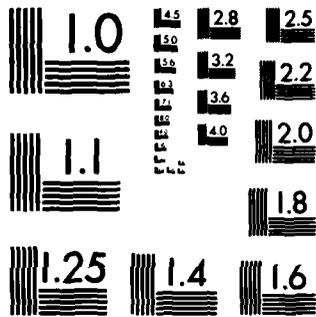
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Carcinogenesis of Nitrated Toluenes and Benzenes, Skin and Lung Tumor Assays in Mice

Final Report

T. J. Slaga
L. L. Triplett
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<p>A series of nitrated toluenes and benzene were tested for their capability to act as initiators, promoters or complete carcinogens in mouse skin. 2,6-dinitrotoluenes and 2-nitrotoluene were found to have weak skin tumor initiating activity. 2,4-dinitrotoluene, 2,6-dinitrotoluenes and 1,3,5-trinitrobenzene produced histological changes in skin which usually are produced by promoting agents; this finding suggests that the three compounds could have skin tumor promoting activity. However, a firm conclusion can only be reached following</p>												

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appropriate in vivo tests. In the lung tumor assay, none of the chemicals tested gave an unequivocal positive response. A borderline positive result for unpurified 2,6-dinitrotoluene could not be repeated when the purified compound was reassayed in the same assay.

19.

Initiation

Lung adenomas

Lung tumors

Mice

4-nitroquinoline-N-oxide

2-nitrotoluene

4-nitrotoluene

Promotion

SENCAR mouse

Skin papillomas

Skin tumors

Strain A mouse

Urethan

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I. Introduction

The wastewaters released into the environment from the 2,4,6-TNT manufacturing process used in US Army munitions plants contain a complex mixture of nitrobenzenes. The compounds in the wastewater are by-products of the manufacturing process or are formed by photolysis. The nitrobenzenes, 1,3-dinitrobenzene (DNE) and 1,2,5-trinitrobenzene (TNB), are produced as by-products or are formed in the environment by photolysis of 2,4-dinitrotoluene (2,4-DNT) and TNT, respectively. Chemical analysis of the wastewaters showed over 30 munitions-related compounds were present. The major components were 2,4-DNT (43 percent), 2,6-DNT (22 percent), and DNB (12 percent) (1). The mutagenicity of the DNT isomers and NBs were investigated with five strains of Salmonella typhimurium with and without activation by postmitochondrial liver fractions in a number of laboratories (2-5). The nitrotoluenes and nitrobenzenes were found to be active in those strains which detect frameshift mutagens. A technical grade mixture composed predominantly of 2,4-DNT (76.5 percent) and 2,6-DNT (18.8 percent) was also shown to be positive in this assay (5).

Two lifetime carcinogenicity studies were conducted with 2,4-DNT and one 104-week study with the technical grade DNT. The National Cancer Institute showed that under the conditions of their bioassay dietary administration of 2,4-DNT to Fischer 344 rats induced benign tumors (fibroma of the skin and subcutaneous tissue in males and fibroadenoma of the mammary gland in females). There was no evidence for the carcinogenicity of the compound in B6C3F1 mice of either sex (6). In a study conducted by the US Army Medical Bioengineering Research and Development Laboratory, Sprague-Dawley rats which received dietary administration of 2,4-DNT for 24 months showed significantly increased incidence of hepatocellular carcinomas and subcutaneous tumors; fibromas in males and mammary fibroadenomas in females (7, 8). Male Swiss mice had a high incidence of renal tumors, including cystic papillary adenomas, solid renal cell carcinomas, and cystic papillary carcinomas. In another study, when the technical grade DNT (75.8 percent 2,4-DNT, 19.5 percent 2,6-DNT, and 4.7 percent other isomers) was fed in the diet, a dose-dependent induction of hepatocellular carcinoma was observed in male and female Fischer 344 rats (9). Male rats had a 100 percent incidence of neoplastic hepatocellular lesions after only one year of DNT feeding; female rats had a 50 percent incidence of similar lesions at the same time.

The earlier onset of overt neoplasms in rats fed the technical grade DNT versus the delayed induction of carcinomas when rats were fed purified 2,4-DNT (>98 percent) suggested that the DNT isomers might act as cocarcinogens or promoters. Since it would be prohibitively expensive to investigate the mechanism of action in lifetime studies, two shorter term in vivo oncogenic bioassays were selected. Strain A mice develop a high incidence of primary lung tumors (adenomas) during their lifetime. The major determinant for the appearance of lung tumors is the age of the animal; older animals have a higher frequency of spontaneous lung tumors than do young animals. The susceptibility of mouse strains to the induction of lung adenomas with chemical carcinogens is in direct relation to the spontaneous occurrence of the tumors in the strain. Therefore,

strain A mice are the most susceptible to a wide variety of carcinogens, in terms of both the earlier appearance and greater multiplicity of tumors (10). The second bioassay used in these studies was the two-stage carcinogenesis system using mouse skin. The SENCAR mouse used in this bioassay was selected for its great sensitivity to 7,12-dimethylbenz(a)anthracene (DMBA)-12-O-tetradecanoylphorbol-13-acetate (TPA) two-stage carcinogenesis. This strain has a low spontaneous incidence of skin papillomas. If putative carcinogens are applied onto the skin of SENCAR mice, followed by repeated application of TPA, the development of tumors may be greatly enhanced and accelerated. The procedure has been found to be a reliable short term assay which will detect complete carcinogens, initiators or promoting agents (11).

A series of nitrotoluenes and nitrobenzenes was examined in the two bioassays to evaluate their usefulness as shorter term carcinogenic models and to elucidate the mechanism of nitrotoluene carcinogenicity.

II. Skin Tumor Studies

1. Objective

The objective of this project was to determine if selected nitrated toluenes and benzenes can induce skin tumors in SENCAR mice by either the initiation-promotion protocol or the complete carcinogenesis protocol.

2. Materials and Methods

a. Materials

The following compounds were tested:

- 2-nitrotoluene (topical initiation)
- 4-nitrotoluene (topical initiation)
- 2,6-dinitrotoluene (topical initiation; i.p. initiation, complete carcinogenesis)
- 2,4-dinitrotoluene (topical initiation; i.p. initiation, complete carcinogenesis)
- 2,6- and 2,4-dinitrotoluene mixture (topical initiation; i.p. initiation; complete carcinogenesis)
- 2,3-dinitrotoluene (topical initiation)
- 2,5-dinitrotoluene (topical initiation)
- 3,4-dinitrotoluene (topical initiation)
- 3,5-dinitrotoluene (topical initiation)
- 1,3,5-trinitrobenzene (topical initiation)
- Benzo(a)pyrene (topical initiation; i.p. initiation; complete carcinogenesis)
- 4-nitroquinoline-N-oxide (topical initiation)
- 7,12-dimethylbenz(a)anthracene (topical initiation)

All agents were dissolved in acetone and solutions were made up fresh before use. The purity of the individual agents was tested as follows. On suitable samples, gas chromatography was performed on a Perkin Elmer Sigma I chromatograph equipped with a 25-m open tubular column (0.2 mm ID) coated with a methyl (5% phenyl) silicone stationary phase. Helium was used as the carrier gas at 1.5 kg/cm² head pressure. The flame ionization detector was operated with hydrogen and air pressures of 1.5 and 2.1 kg/cm², respectively. Mass spectra were obtained with a Hewlett-Packard 5985A gas chromatograph/mass spectrometer. Electron impact spectra were obtained at 70 eV at an ion source temperature of 200°C. The gas chromatograph interfaced to the spectrometer was operated using conditions similar to those described above. Nuclear magnetic resonance spectra were obtained with a JEOL FX90Q spectrometer at 89.56 MHz for proton observation and 22.50 MHz for carbon-13. Silica gel plates were used for the thin layer chromatography studies.

b. Methods

The dose levels were derived from the results of the toxicity studies, the amount of compound that could be dissolved in 0.2 ml of acetone and the availability of the compound. In the prechronic toxicity studies a dose was chosen that did not kill any of the animals during a one-week period. In addition, the ability of the various compounds to induce inflammation and hyperplasia in the skin was determined. Tumors were counted once a week, and body weights were recorded every two weeks during the skin carcinogenesis studies. The tumor types were verified histologically at the end of the tumor experiments.

c. Experimental Design:

The nitrated toluenes and benzenes were first tested to determine if they were cytotoxic by applying various dose levels to the skin. Next, the various compounds were tested to determine if they induced any morphological changes in the skin. After the prechronic studies, the various compounds were tested to determine if they had tumor initiation activity in an initiation-promotional protocol using TPA as the promoter. Although all the compounds were tested as initiators by topical application, 2,6- and 2,4-dinitrotoluene and a mixture of the two were also tested by intraperitoneal injection followed by topical application with TPA. In addition, 2,4- and 2,6-dinitrotoluene were tested as complete skin carcinogens by giving them once a week for 52 weeks. In the tumor initiation studies, the nitrated toluenes and benzenes were given once followed by once weekly promotion with 4 µg of TPA for 30 weeks. In all experiments both papillomas and squamous cell carcinomas were quantitated. The papilloma data is expressed as percentage of mice with papillomas and as the average number of papillomas per mouse. The carcinoma data are expressed as the percentage of mice with squamous cell carcinomas.

3. Results

a. Chemical Analysis of Nitrotoluenes

The eleven nitro-substituted aromatics were tested for purity and compound verification in support of biological testing. The purities of the compounds were first tested by comparing the melting points of the materials received versus values reported in the literature. Gas chromatography using a high resolution capillary column was employed to obtain a more quantitative estimate of compound purity. Thin layer chromatography was used to test for the presence of any non-gas chromatographable impurities. The identities of the materials and their major impurities were verified using a variety of spectroscopic techniques including combined gas chromatography/mass spectrometry and carbon-13 and proton nuclear magnetic resonance spectroscopy.

The purities of all the compounds tested were 98% or greater except for one lot of 2,4-dinitrotoluene (8/19/80) and several lots of 2,6-dinitrotoluene. The 2,6-dinitrotoluene from Pfaltz & Bauer (5/28/80) was purified on a silica gel column to a level of at least 99%. A summary of the tests on the nitroaromatics is given in Table 1.

b. Skin Painting Studies

In general, all of the nitrated toluenes and benzenes were relatively non-toxic in the prechronic studies. In terms of induction of inflammation and hyperplasia, 2,4-dinitrotoluene, 2,6-dinitrotoluene and 1,3,5-trinitrobenzene were very effective whereas all of the other nitrated toluenes were negative. 1,3,5-Trinitrobenzene was as active as the potent tumor promoter TPA in inducing inflammation, hyperplasia and dark basal keratinocytes. These events (especially dark cells) seem to correlate with the promoting activity of tumor promoters (12). 2,4- and 2,6-dinitrotoluene were active but less than for 1,2,5-trinitrobenzene. Three dose levels of 2,4-dinitrotoluene (1, 5 and 10 mg), 2,6-dinitrotoluene (1, 5 and 10 mg) and 1,3,5-trinitrobenzene (2, 10 and 50 mg) were applied topically to mice. The animals were killed 1, 2, 4 and 6 days later in order to determine the effects of these compounds on epidermal hyperplasia and dark cells. We used four mice per time point; only the two higher doses of 2,4- and 2,6-dinitrotoluene gave a significant level of epidermal hyperplasia and dark cells. The response of 2,4- and 2,6-dinitrotoluene at 10 mg was similar to the response of 1 µg of TPA. All three doses of 1,3,5-trinitrobenzene gave a significant hyperplastic and dark cell response. Dose levels of 10 and 50 mg of 1,3,5-trinitrobenzene gave a hyperplastic and dark cell response similar to the maximum response obtained with TPA. Because of these results, these compounds should be tested as skin tumor promoters.

The tumor results for the various nitrated toluenes and benzenes are summarized in Tables 2-4. In all groups, mice survival was 90% or more, except for animals treated with benzo(a)pyrene for 52 weeks. In addition, all mice continued to gain weight during exposure to the test compounds. All group weights determined at the end of the experiment period were well

TABLE 1. SUMMARY OF ANALYSES OF NITROTOLUENES AND RELATED COMPOUNDS

Compound	Source	Melting Point, °C		Purity, %	Identification				Impurities Found
		Found	Literature		GC/MS	¹³ C NMR	¹ H NMR		
3,5-Dinitroaniline	A	160-161	160-162	>99	X	X	X	X	none
1,3-Dinitrobenzene	BDH	90.5	89.8	99	X	X	X	X	4-nitrotoluene
1,3,5-Trinitrobenzene	SRI	122-123	121-122.5	>99	X	X	X	X	none
2-Nitrotoluene	E	(liquid)	-	98	X	X	X	X	3-nitrotoluene
4-Nitrotoluene	E	-	-	98	X	X	X	X	2- and 3-nitrotoluene
2-3-Dinitrotoluene	A	59.5-60	59-61	98	X	X	X	X	2,5-dinitrotoluene
2,4-Dinitrotoluene	NA	67-70.5	69-70	98	X	X	X	X	2-nitrotoluene, 2,6-dinitrotoluene
	MC&B	69.5-71	69-70	92-95	X	X	X	X	2,6-dinitrotoluene
2,5-Dinitrotoluene	ICN	49-50.5	52.5	>99	X	X	X	X	none
2,6-Dinitrotoluene	NA	57.5-59	64-66	92-95	X	X	X	X	2-nitrotoluene, 2,4-dinitrotoluene
	A	63-65.5	"	98	X	X	X	X	none
	P&B	52	"	92-95	X	X	X	X	2-nitrotoluene, 2,4-dinitrotoluene
	Purified P&B	6/13/80		>99	X	X	X	X	none
3,4-Dinitrotoluene	A	57-59	54-57	>99	X	X	X	X	none
3,5-Dinitrotoluene	SRI	91-92.5	92-93	99	X	X	X	X	none

A: Aldrich
 E: Eastman
 MC&B: Matheson, Coleman & Bell
 NA: Not available
 P&B: Pfaltz & Bauer
 SRI: Synthesized and provided by Ronald Spanggard of SRI, International

within the normal range found historically for our outbred SENCAR stock and no significance can be attributed to eventual differences in group weights. All of the positive control experiments (BP-TPA, NQO-TPA, DMBA-TPA and BP complete carcinogenesis studies) gave responses of similar magnitude as found in our historical controls and in previous experiments (11). This indicated that the assays of the nitrated toluenes and benzenes were valid. In some experiments the TPA only treated mice developed a low level of papillomas and carcinomas.

The following compounds were found to be negative as skin tumor initiators: 4-nitrotoluene; 2,4-dinitrotoluene; 2,3-dinitrotoluene; 2,5-dinitrotoluene; 3,4-dinitrotoluene; 3,5-dinitrotoluene and 1,3,5-trinitrobenzene. These compounds did not give a tumor response above that seen with the TPA only treated mice. 2-Nitrotoluene at 240 mg and 2,6-dinitrotoluene at 10 mg given topically produced a somewhat higher number of mice carrying a carcinoma than seen in all the other groups (except for the positive controls) (Table 2). However, the incidence of tumor bearing animals, evaluated with a X^2 -square test, was not statistically significant ($p > 0.05$). When the average number of papillomas per mouse in the two treatment groups was compared to animals treated with TPA alone (t-test), the difference was statistically not significant ($p > 0.05$). 2,6-Dinitrotoluene, when given intraperitoneally at a dose of 10 mg/kg and evaluated as a tumor initiator, produced a 10% incidence of carcinoma bearing mice; again this was statistically not significant ($p > 0.05$) (Table 3). When the average number of papillomas per mouse for 2-nitrotoluene and 2,6-dinitrotoluene were compared to the average number of papillomas found in animals treated with TPA alone, the difference (estimated by t-test) was statistically not significant. While this allows to exclude a false positive response, it does not allow to exclude a false negative one.

Likewise, both a 1:1 and 2:1 mixture of 2,5-dinitrotoluene and 2,6-dinitrotoluene were negative as skin tumor initiators when given topically or intraperitoneally. It should be pointed out that the topical application of the 1:1 mixture gave a weak papilloma response but did not give any carcinomas. Furthermore, 2,4-dinitrotoluene and 2,6-dinitrotoluene were negative as complete skin carcinogens when given topically once a week for 52 weeks (Table 4).

4. Conclusion

It is concluded that 2-nitrotoluene and 2,6-dinitrotoluene might have weak skin tumor initiating activity because they gave a higher papilloma and carcinoma response than did TPA only treated mice, whereas no such evidence was found for the other nitrated toluenes and benzenes. It should be pointed out that the histological studies suggest that 2,4-dinitrotoluene, 2,6-dinitrotoluene and 1,3,5-trinitrobenzene might have skin tumor promoting activities. The hyperplastic and dark cell response of 1,3,5-trinitrobenzene at the two highest doses was similar to the maximum response obtainable with TPA, whereas 2,4- and 2,6-dinitrotoluene gave a response at the two highest doses similar to that of 1 μ g of TPA. These

TABLE 2. TUMOR RESPONSE AT 30 WEEKS WHEN GIVEN AS A TUMOR INITIATOR TOPICALLY

Initiator	Dose	Papillomas per mouse	Group weight beginning (g)	Group weight end (g)	% of Mice with Papillomas	% of Mice with Carcinomas
Benzo(a)pyrene (BP)	50 mg	6.4 ± 1.10	32.4	35.6	100	32
	25 mg	5.3 ± 0.85	33.7	38.7	100	16
	12.5 mg	2.5 ± 0.44	33.0	39.4	72	9
4-Nitro- quinoline- oxide	1 mg	2.1 ± 0.46	31.0	37.8	72	12
	0.5 mg	1.6 ± 0.37	32.0	36.8	62	9
	0.1 mg	1.1 ± 0.32	34.0	37.7	52	5
4-NT	400 mg	0.14 ± 0.07	34.2	37.8	11	2.5
	250 mg	0.10 ± 0.05	34.6	40.0	10	-
	50 mg	0.10 ± 0.07	34.0	38.9	10	-
2-NT	240 mg	0.22 ± 0.09	32.2	37.2	16	5.0
	120 mg	0.13 ± 0.06	33.4	38.0	10	2.5
	24 mg	0.03 ± 0.01	33.2	36.9	2.5	-
2,6-DNT	10 mg	0.20 ± 0.02	32.8	39.6	20	5.0
	5 mg	0.10 ± 0.07	32.7	40.1	7	0
	1 mg	0.15 ± 0.02	30.5	39.6	15	0
2,4-DNT	10 mg	0.05 ± 0.01	33.5	40.5	5	0
	5 mg	0.05 ± 0.01	33.4	41.7	5	0
	1 mg	0.07 ± 0.02	32.7	39.0	7	0
2,4-DNT + 2,6-DNT (1:1)	10 mg	0.08 ± 0.01	33.1	40.1	8	0
	5 mg	0.18 ± 0.04	32.0	39.6	15	0
	1 mg	0.15 ± 0.03	32.5	43.5	15	0
2,4-DNT + 2,6-DNT (2:1)	10 mg	0.15 ± 0.03	33.2	40.0	15	0
	5 mg	0.10 ± 0.02	32.0	38.1	10	0
	1 mg	0.07 ± 0.01	33.1	39.2	7	0
2,3-DNT	50 mg	0.07 ± 0.02	35.3	42.2	7	0
	10 mg	0.10 ± 0.02	33.8	40.6	10	0
	2 mg	0.07 ± 0.01	35.9	44.0	7	0
2,5-DNT	50 mg	0.05 ± 0.01	36.9	44.1	5	0
	10 mg	0.05 ± 0.01	35.6	42.2	5	0
	2 mg	0.07 ± 0.02	35.1	43.6	7	0

(TABLE 2 continued)

TABLE 2 (continued)

3,4-DNT	50 mg	0.07 ± 0.01	34.3	41.3	7	0
	10 mg	0.00 ±	37.0	44.2	0	0
	2 mg	0.05 ± 0.01	36.0	42.0	5	0
3,5-DNT	50 mg	0.00 ±	31.6	38.0	0	0
	10 mg	0.05 ± 0.01	36.0	42.4	5	0
	2 mg	0.05 ± 0.02	34.6	41.9	5	0
1,3,5-TNB	50 mg	0.12 ± 0.02	35.6	40.8	5	0
	10 mg	0.05 ± 0.01	33.0	37.4	5	0
	2 mg	0.05 ± 0.01	32.2	37.7	5	0
TPA only	4 µg	0.18 ± 0.04	34.3	42.1	13	2.5

TABLE 3. TUMOR RESPONSE AT 30 WEEKS WHEN GIVEN AS A TUMOR INITIATOR
INTRAPERITONEALLY

Initiator	Dose	Papillomas per Mouse	Group weight beginning (g)	Group weight end (g)	% of Mice with Papillomas	% of Mice with Carcinomas
2,6-DNT	10 mg	0.22 ± 0.04	32.4	41.8	19	10
	5 mg	0.10 ± 0.02	33.5	41.5	10	2.5
	1 mg	0.08 ± 0.02	32.3	40.1	8	0
2,4-DNT	10 mg	0.07 ± 0.01	32.0	39.8	7	0
	5 mg	0.05 ± 0.01	33.5	39.7	5	0
	1 mg	0.07 ± 0.02	31.9	40.5	7	0
2,4-DNT + 2,6-DNT (2:1)	10 mg	0.08 ±	33.5	38.8	8	0
	5 mg	0.18 ±	32.0	37.7	15	0
	1 mg	0.15 ±	31.8	37.0	15	0
BP	50 mg	1.8 ± 0.35	33.0	38.0	60	22
	25 mg	0.8 ± 0.12	32.9	39.2	28	16
	12.5 mg	0.5 ± 0.09	31.5	35.8	22	12
Corn oil	--	0.05 ± 0.01	32.0	39.4	8	0

TABLE 4. COMPLETE CARCINOGENIC ACTIVITY OF 2,4-DNT AND 2,6-DNT AT 52 WEEKS

Compound	Dose (1X/wk)	Group weight beginning (g)	Group weight end (g)	% of Mice alive at 52 weeks	% of Mice with Papillomas	% of Mice with Carcinomas
2,6-DNT	10 mg	29.7	40.1	95	0	0
	5 mg	31.0	39.6	98	0	0
	1 mg	31.4	43.3	100	0	0
2,4-DNT	10 mg	30.4	41.1	96	0	0
	5 mg	32.4	38.8	98	0	0
	1 mg	32.8	41.9	98	0	0
BP	50 mg	35.1	-	0	100	100
	25 mg	30.4	40.1	42	47.5	80
	12.5 mg	34.1	39.6	60	10	62

compounds are good candidates for testing as tumor promoters in the skin assay.

III. Lung Tumor Studies

1. Objective

The objective of the project was to determine if multiple intraperitoneal injections of eight selected nitrated toluenes and one nitrated benzene could induce lung tumors in young strain A/Jax male mice.

2. Materials and Methods

a. Materials

The following compounds were tested: (see Appendix for source)

2-nitrotoluene
4-nitrotoluene
2,3-dinitrotoluene
2,4-dinitrotoluene
2,5-dinitrotoluene
2,6-dinitrotoluene (purified and unpurified)
3,4-dinitrotoluene
3,5-dinitrotoluene
A mixture of 2:1, 2,4-dinitrotoluene and 2,6-dinitrotoluene
1,3,5-trinitrobenzene
Urethan (ethyl carbamate)
Benzo(a)pyrene
4-nitroquinoline-1-oxide
Corn oil (vehicle)

The purity of the materials was the same as described in section II. All substances except urethan were dissolved in corn oil, and solutions were prepared fresh every week. Urethan was dissolved in 0.15 M NaCl solution.

b. Methods

The procedure adopted was the one originally recommended by Shimkin and Stoner (13). In preliminary toxicity studies, the maximum tolerated dose (MTD) for each compound was determined. The MTD is defined as the highest dose that results in no mortality (5 mice per dose) when injected three times a week for two consecutive weeks. For the lung tumor assay itself, each substance was injected i.p. into groups of mice three times a week (Monday, Wednesday, Friday) for eight consecutive weeks. The following doses were tested: the MTD, one-half the MTD, and one-fifth the MTD. Control groups were: (1) mice injected with corn oil (vehicle control); (2) mice given a single injection of urethan at 1 g/kg body weight

(positive calibration control; and (3) mice that received nothing. Body weights were recorded every two weeks and the mice were killed 16 weeks after the last injection. The lungs were fixed in Tellyesniczky's solution, and tumors on the lung surface were counted.

c. Experimental design

The mice were injected with the test substance three times a week for eight consecutive weeks. The animals were killed 16 weeks after the last injection, and lung tumors were counted. Indices of tumorigenicity were tumor incidence (the number of tumor bearing mice per group) and tumor multiplicity (the average number of tumors per lung).

d. Statistical analysis

Data were subjected to statistical evaluation using the two-tailed Student t-test for tumor multiplicity and Chi-square analysis for tumor incidence. Examination of the data indicates that these simple statistical parameters were probably sufficient as a means of evaluating the tumorigenic potential of the chemicals tested in this assay. In all experiments levels of statistical significance, to reject the Null-hypothesis, were set a priori at a level of $p < 0.05$.

3. Results

Four separate assays were conducted to determine the lung tumorigenicity of the chemicals. The results are presented in Tables 5-8 together with the corresponding control data.

In all four assays, the average body weight of the animals increased about 30% in all groups during the 24-week period. There was no indication of a treatment related effect on body weight for any chemical.

The lung tumor incidence for mice given a single injection of urethan was 100% and the multiplicity was about one lung tumor per mg of urethan injected. These results show that the mice in each experiment had responded appropriately to the positive calibration control carcinogen (13).

An apparent dose response for incidence was observed for 4- and 2-nitrotoluene, for 2,3-, 2,4-, and purified 2,6-dinitrotoluene, and for multiplicity for 4-nitrotoluene and purified 2,6-dinitrotoluene. But a statistical evaluation of the data showed that none of these chemicals at any dose evoked a tumor response significantly above that of the corn oil control groups at a p level of 0.05 or less. The sole indication of a positive response was obtained with the high dose of unpurified 2,6-dinitrotoluene (Table 5) which produced an increase in both incidence and multiplicity over the corn oil control group at a significance level of $0.05 < p < 0.1$. This suggestive, borderline evidence was not evident from

TABLE 5. LUNG TUMOR ASSAY FOR 4-NITROTOLUENE AND 2,6-DINITROTOLUENE (ASSAY 01-14-80)

Substance	Total dose (mg/kg)	No. initial	No. at end	Survival (%)	Group weight begin (g)	Group weight end (g)	Survivors with tumors (%)	No. of ^a tumors/lung ± SE
4-Nitrotoluene	9000	30	29	97	20.4	28.1	24	0.24 ± 0.08
	4500	30	29	97	18.7	26.4	14	0.21 ± 0.11
	1800	30	26	87	21.0	27.2	12	0.12 ± 0.07
2,6-Dinitrotoluene ^b	3600	30	24	80	21.7	26.8	38	0.50 ± 0.16
	1800	30	28	93	21.1	27.9	21	0.29 ± 0.12
	720	30	28	93	21.5	26.0	23	0.35 ± 0.15
Corn oil	24 × 10 ⁴	50	47	94	19.8	26.6	17	0.19 ± 0.07
Urethan ^c	1000	50	50	100	20.8	26.7	100	22.12 ± 1.36 ^d
Nothing	—	50	50	100	20.3	25.9	16	0.18 ± 0.06

^aMean ± SE

^bUnpurified

^cSingle injection: 1000 mg/kg

^dp < 0.05 compared to corn oil or nothing controls

TABLE 6. LUNG TUMOR ASSAY FOR 2-NITROTOLUENE AND 2,4-DINITROTOLUENE (ASSAY 04-14-80)

Substance	Total dose (mg/kg)	No. initial	No. at end	Survival (%)	Group weight begin (g)	Group weight end (g)	Survivors with tumors (%)	No. of tumors/lung ± SE
2-Nitrotoluene	6000	30	23	77	20.6	27.8	17	0.17 ± 0.08
	3000	30	27	90	21.4	28.1	11	0.15 ± 0.09
	1200	30	29	97	20.0	27.4	10	0.10 ± 0.06
2,4-Dinitrotoluene	1200	30	30	100	19.6	26.7	17	0.17 ± 0.07
	600	30	29	97	20.3	26.7	14	0.17 ± 0.09
	240	30	27	90	19.4	26.9	0	0
Corn oil	24 × 10 ⁴	20	17	85	19.5	27.8	6	0.06 ± 0.06
Urethan ^a	1000	50	46	92	21.2	26.2	100	24.52 ± 1.09
Nothing	---	50	48	96	21.7	27.0	23	0.27 ± 0.08

^aSingle injection 1000 mg/kg.

TABLE 7. LUNG TUMOR ASSAY FOR 2,3-DINITROTOLUENE, 2,5-DINITROTOLUENE, 2,6-DINITROTOLUENE AND A MIXTURE OF 2,4- AND 2,5-DINITROTOLUENE (ASSAY 03-09-81)

Substance	Total dose (mg/kg)	No. initial	No. at end	Survival (%)	Group weight begin (g)	Group weight end (g)	Survivors with tumors (%)	No. of tumors/lung \pm SE
2,3-Dinitrotoluene	4800	30	26	87	19.9	26.2	35	0.38 \pm 0.11
	2100	30	27	90	19.5	26.0	30	0.30 \pm 0.09
	840	30	15	50	19.4	24.3	20	0.20 \pm 0.11
2,5-Dinitrotoluene	4800	30	16	53	18.1	24.3	13	0.13 \pm 0.09
	2400	30	18	60	20.4	26.3	24	0.47 \pm 0.27
	960	30	27	90	20.6	25.5	7	0.07 \pm 0.05
2,6-Dinitrotoluene (purified)	4800	30	3	10	17.6	22.0	33	0.33 \pm 0.33
	2400	30	28	93	19.7	25.6	18	0.21 \pm 0.10
	960	30	27	90	19.4	26.5	7	0.07 \pm 0.05
2,4-2,6-Dinitrotoluene (2:1)	2400	30	24	80	18.9	24.1	33	0.42 \pm 0.13
	1200	30	29	97	19.7	26.0	21	0.52 \pm 0.35
	480	30	26	87	19.7	26.8	15	0.19 \pm 0.10
Corn oil ip	24 x 10 ⁴	50	41	82	19.6	26.0	27	0.29 \pm 0.08
Urethan ^a	1000	20	19	95	19.2	25.3	100	21.16 \pm 1.92
Nothing	—	30	24	80	18.5	25.7	33	0.46 \pm 0.15

^aSingle injection i.p. 1000 mg/kg.

TABLE 8. LUNG TUMOR ASSAY FOR 3,4-DINITROTOLUENE, 3,5-DINITROTOLUENE, and 1,3,5-TRINITROZENE (ASSAY 06-15-81)

Substance	Total dose (mg/kg)	No. initial	No. at end	Survival (%)	Group weight begin (g)	Group weight end (g)	Survivors with tumors (%)	No of tumors/lung \pm SE
3,4-Dinitrotoluene	6000	40	8	20	18.7	21.8	25	0.25 \pm 0.17
	3000	30	17	57	18.7	21.9	12	0.18 \pm 0.13
	1200	30	16	53	18.9	25.0	19	0.19 \pm 0.10
3,5-Dinitrotoluene	6000	30	1	3	19.5	29	0	--
	3000	30	22	73	18.6	24.5	14	0.14 \pm 0.07
	1200	30	14	47	19.6	24.1	7	0.07 \pm 0.07
1,3,5-Trinitrobenzene	3000	30	15	50	19.2	24.1	13	0.13 \pm 0.09
	1500	30	14	47	18.9	24.9	14	0.14 \pm 0.10
	600	30	18	60	18.5	25.8	6	0.06 \pm 0.06
Corn oil	24×10^4	50	35	70	19.5	26.6	9	0.09 \pm 0.05
Urethan	1000	20	10	50	18.8	25.1	100 ^a	25.10 \pm 2.66
Nothing	--	30	18	60	19.1	25.6	11	0.11 \pm 0.08

^aSingle i.p. injection 1000 mg/kg.

TABLE 9. LUNG TUMOR ASSAY FOR BENZO(A)PYRENE AND 4-NITROQUINOLINE-N-OXIDE (ASSAY 07-28-8 and 10-20-80)

Substance	Total dose (mg/kg)	No. initial	No. at end	Survival (%)	Group weight begin (g)	Group weight end (g)	Survivors with tumors (%)	No. of tumors/lung ± SE
Benzo(a)pyrene (Assay 07-28-80)	12	30	19	63	19.7	28.0	26	0.30 ± 0.10
	6	30	28	93	20.3	27.0	21	0.30 ± 0.10
	2.4	30	20	67	20.6	26.5	20	0.20 ± 0.09
Corn oil	24 × 10 ⁴	30	24	80	20.6	27.3	29	0.30 ± 0.10
Urethan	1000	20	17	85	22.2	26.9	100 ^a	17.18 ± 2.00
Nothing	----	20	20	100	21.4	26.3	15	0.30 ± 0.20
4-Nitroquinoline-N- oxide (Assay 10-20-80)	30	30	19	63	20.2	24.3	42	0.74 ± 0.24
	15	30	15	50	20.1	24.8	20	0.27 ± 0.15
	6	30	20	66	18.8	24.2	25	0.30 ± 0.13
Corn oil	24 × 10 ⁴	30	16	53	19.2	24.4	31	0.31 ± 0.12
Urethan	1000	20	17	85	19.9	25.0	100 ^a	19.41 ± 1.55
Nothing	----	20	14	70	20.2	22.9	21	0.36 ± 0.20

^aSingle i.p. injection 1000 mg/kg.

test results with purified 2,6-dinitrotoluene (Table 7) although interestingly there was an apparent but not significant dose response for lung tumor incidence and multiplicity in mice given this preparation.

Benzo(a)pyrene and 4-nitroquinoline-N-oxide were the two compounds used in the skin tumor assay as positive controls. They also were evaluated in the lung tumor assay. The data are given in Table 9. Benzo(a)pyrene, injected repeatedly i.p. at cumulative doses allowing at least 60% survival of the animals was completely ineffective as a mouse lung carcinogen. 4-Nitroquinoline-N-oxide produced a tumor incidence of 42% at the highest dose given and a tumor multiplicity of 0.74 tumors per mouse. While these values were higher than that of any chemical tested (other than urethan), the increase over values for concomitant corn oil control groups gave p-values of 0.13 for multiplicity and 0.51 for incidence. This was not enough to be considered a significant response.

In two of the assays (Nos. 03-09-81, Table 7 and 06-15-81, Table 8) there was an outbreak of Citrobacter freundii infection in our experimental animals. The disease produces hyperplasia of the colonic mucosa and rectal prolapse and may be on occasion a problem in bioassay (14-16). While Citrobacter infection did not seem to produce undue mortality in assay 03-09-81 (Table 7), we nevertheless instigated prophylactic treatment with neomycin sulfate. The animals were given 2 mg/ml of neomycin in the drinking water during 24 hours in the third, fourth and eighteenth week of the assay. In assay 06-15-81 (Table 8) Citrobacter infection seemed to cause unduly high mortality (survival around 50% only). In this experiment, animals were treated for 48 hours with neomycin (2 mg/ml) for 2 days in the first and third week of the assay.

It must be pointed out at this time, that in these two "problem" assays both positive (urethan) and vehicle (corn oil) controls gave tumor incidence and multiplicity in the anticipated range.

4. Conclusions

Before interpreting our data a few words should be said on how they have been analyzed. We have followed the criteria developed by Shimkin and Stoner (13). They can be summarized as follows:

- a. In untreated control mice, tumor multiplicity should approximate the anticipated level (0.20 tumors/lung). In mice given 1000 mg/kg of urethan ip, usually 1 tumor per 1 mg of urethan should be found.
- b. Whether a carcinogenic response has been elicited depends on whether the number of tumors per lung in treated animals is significantly different ($P < 0.05$ as determined by a simple t-test) from the number of tumors per lung in vehicle-treated controls.

c. A test is positive if:

- (1) Treated animals have significantly more tumors per lung than controls, preferably one or more tumors per mouse.
- (2) There is a dose-response relationship.
- (3) Tumor multiplicity in vehicle-treated controls is within the anticipated range and not unduly low.

In the analysis of the data we have adhered strictly to these criteria. In addition, we have on occasion compared incidence of tumors between different groups by Chi-square analysis. A more sophisticated analysis of the data could be done if necessary, but at the present time the additional effort does not seem warranted.

In our controls, the mean number of tumors per lung in the untreated animals was 0.24 with a range of 0.11 to 0.46. The mean tumor incidence for untreated animals was about 18%. Animals given a total of 24 injections of corn oil had a lung tumor incidence and multiplicity comparable to values observed for untreated mice. The average number of tumors per lung that resulted from a single injection of urethan at 1000 mg/kg was 22.6 ± 1.4 with a range of 17.2 to 25.1.

The data for our control animals generally compare well with control data compiled by Shimkin and Stoner (13). However, in two assays (04-14-80 and 06-15-81) the values for tumor incidence and multiplicity in corn oil-treated animals must be considered abnormally low. In the positive controls — animals treated with 1000 mg/kg of urethan — the tumor response was usually the one we would have expected from both the data provided by Shimkin and Stoner (13) and from some of our own experiments on BHT-enhanced tumor formation in the lungs of A/Jax mice (17, 18).

In conclusion, our control data compare favorably with historical controls. Nevertheless, during the 3 year period there were instances of considerable variation both in untreated animals and in animals given a standard dose of urethan. We would therefore recommend that comparisons should be made within a given assay only, provided control values approximate those found in previous or later assays. We would fully agree with the statement by Shimkin and Stoner (13) that "occasionally, statistical significance is obtained because control animals demonstrate fewer than the anticipated number (of tumors), and it is obviously not acceptable data on which to conclude a positive effect."

If we analyze our data accordingly, then we have to conclude that none of the chemicals tested in the mouse lung tumor assay gave an unequivocally positive response. It appears justified to discount the borderline positive results observed for unpurified 2,6-dinitrotoluene: statistical significance at a level of 0.05 was not achieved. Furthermore, the repeat experiment with purified 2,6-dinitrotoluene was unequivocally negative.

Unfortunately, the negative data obtained in the lung tumor assay do not allow us to ascertain that any of the compounds tested would be devoid of carcinogenic potential. This for three reasons:

1. The conclusion to be drawn for "negative" compounds must be limited to the dose to which animals were exposed and to other conditions of the experiment (13). Negative data in the lung tumor assay are thus not conclusive for absence of carcinogenic potential in a different system. It is however reassuring that, with the present series of compounds, the skin tumor studies also showed low, if any, carcinogenic potential.
2. Two of our assays were plagued by Citrobacter infection. The necessary treatment with antibiotics might have altered the metabolism of the nitroaromatics and thus their carcinogenicity. We consider this to be unlikely, however. Treatment with neomycin was for 24 or 48 hours only, whereas treatment with carcinogens was 3 times a week, for 8 weeks. It does not seem very likely that neomycin given for 24-48 hours in the drinking water would have such effects as to radically alter carcinogen metabolism and to suppress all carcinogenic potential.

We also have found, in a series of experiments reported elsewhere, that the tumor response to 8 different compounds is virtually the same regardless of whether animals are infected with Citrobacter and treated with neomycin or not (19). We are confident that Citrobacter infection and neomycin treatment did not influence the testing of compounds listed in Tables 7 and 8, although we arrive at this conclusion by inference only, and not by experimentation.

3. The potentially most disturbing observation made in this study was that both benzo(a)pyrene and 4-nitroquinoline-N-oxide were negative in the lung tumor assay (Table 9). The same two compounds were used as positive controls in the skin tumor studies (Tables 2-4), where they elicited a good response.

The two compounds were used in the lung tumor assay with the explicit purpose to compare eventually data between the skin system and the lung system with regard to relative potency. The failure to observe a positive response in the lung system does not allow us to do this. Moreover, the observation raises the distinct possibility that the lung tumor assay, as it was done in this study, is not always an appropriate and reliable screening test for carcinogens. A recently completed large study, involving more than 40 compounds, lends support to this conclusion (19).

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APPENDIX

Source of chemicals

1. 2-nitrotoluene - Eastman Kodak Co., lot A5F
2. 4-nitrotoluene - Eastman Kodak Co., lot B7A
3. 2,3-dinitrotoluene - Aldrich Chemical Co., lot 3317DE
4. 2,4-dinitrotoluene - Matheson, Coleman and Bell Co., lot L6H09
5. 2,5-dinitrotoluene - ICN Pharmaceutical, K and K Labs Division, lot 13745
6. 2,6-dinitrotoluene - Aldrich Chemical Co., lot 031947 (unpurified). Pfaltz and Bauer Chemical Co., lot D48070 (purified and for mixture with 2,4-dinitrotoluene).
7. 3,5-dinitrotoluene - Aldrich Chemical Co., lot CB 082467
8. 3,5-dinitrotoluene - R. J. Spangord, SRI International. Special lot purchase.
9. 1,3,5-trinitrobenzene - R. J. Spangord, SRI International. Special lot purchase.
10. Urethan (ethyl carbamate) - Sigma Chemical Co., lot 116C-0291
11. Corn oil - Eastman Kodak Co., lots D-4-25 and D445; Fisher Scientific Co., lot 781672.
12. Benzo(a)pyrene - Sigma Chemcial Co., lot 15C-0116
13. 4-nitroquinoline-1-oxide - ITT Research Institute, lot ET 4-82-1

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